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ScienceDirect

Procedia Food Science 7 (2016) 5 – 8

Procedia Food Science

9th International Conference on Predictive Modelling in Food

Sequencing and identification of different *Salmonella* species in cocoa beans treated with gamma irradiation

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Abstract

Gamma irradiation is an effective way to eliminate the bacterial load in cocoa beans. During harvesting these are exposed to various factors of contamination. The aim of this study was to analyze the effect of gamma irradiation on the presence of *Salmonella spp* considered a microbiological risk in chocolate. Cocoa samples (n = 31) were treated with three doses of gamma irradiation (2, 3 and 5 kGy) besides a control sample without irradiation. The PCR method revealed 22 bacteria (n = 124) with Salm3-Salm4 amplified. The DNA sequencing method confirmed the presence of two isolates who belong to *Salmonella spp.* and 20 belonging to *Klebsiella sp* and *Enterobacter sp*. These results were retrieved from the control samples and irradiation 2kGy, while radiation 3 to 5 kGy no growth of microorganisms.

Peer-review under responsibility of Department of Food Science, Faculty of Food Engineering, University of Campinas.

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Peer-review under responsibility of Department of Food Science, Faculty of Food Engineering, University of Campinas.
doi:10.1016/j.profoo.2016.02.074

Keywords: Irradiation; cocoa beans; *Salmonella* spp; PCR specifc group; 16S sequencing.

1. Introduction

Cocoa beans (*Theobroma cacao L.*) is the main raw material for the production of chocolate. During the primary processing, the fruits are harvested and opened with knives, to separate the seeds with pulp that are fermented in wooden boxes, for example. This is, one of the most important stages of cocoa processing[1,2]. During this stage, the cocoa seeds are constantly handling. The aeration of the mass, the conditions of room temperature, pH and acidity of the pulp contribute to the growth of specific microorganisms[3]. These microorganisms will play a role in the sensory aspect and quality of chocolate, but also a problem of food security, since the growth of pathogenic microorganisms and transmitter's foodborne diseases. Studies show that during the pre - processing of cocoa is no further contamination[4,5].

Salmonella is the leading known microbiological risk in chocolate. Its origin is not allocated to the cocoa or the environment where it grows, it is of fecal origin. It can be introduced from the hands of the workers during handling. The only stage at which is possible to eliminate the presence of *Salmonella* is the roasting of the beans or nibs. Subsequently, it is important to minimize the risk of contamination after this step because there is not another stage to be eliminated [1,6]. Da Silva demonstrated the presence of total *Enterobacteriaceae*, coliforms, *Escherichia coli* and *Salmonella* in chocolates produced in Brazil [4]. Although the aw and pH values are unsuitable for *Salmonella* survival, it can survive in those conditions. Moreover the high fat content in the chocolate (> 20%) increases the heat resistance of the *Salmonella* [6,7,8].

Food irradiation can control microbial development, contributes in reducing the incidence of some diseases, it will increase the shelf life of food [10]. They were used gamma irradiation sources cobalt 60, the main action of ionizing radiation. It occurs through changes in genetic material, this will depend on the intensity of treatment and the type of microorganism. Gram-positive bacteria are more tolerant to irradiation that Gram-negative bacteria [11]. Studies show that cocoa beans from Ghana, need a dose of 5 kGy to reduce the microbial burden [12]. In this context, this paper aims to contribute with necessary information regarding the use of this technique, controlling the growth of *Salmonella* in cocoa.

2. Materials and methods

2.1 Irradiation Test

Were analyzed 31 batches of cocoa beans the pre-processing stage. Each batch was subdivided into 4 subgroups. They were packed in polyethylene bags and were irradiated with Cobalt-60 source underwent to three doses of irradiation (2, 3 and 5kGy) and a control without irradiation in CBE (Companhia Brasileira de Esterilização) - São Paulo, Brazil. Furthermore, the samples were stored at 7 °C in the Microbiology Division of the Research Center for Chemistry, Biology and Agriculture - CPQBA/UNICAMP.

2.2 Isolation of *Salmonella*

It was performed by the method of the Food and Drug Administration using reference methods in the Manual for "Microbiological Analysis Methods of Food and Water" [13]. The pre-enrichment was performed with the milled cocoa beans and suspended in buffered peptone water (BPW), controlling pH 6.8 +/- 0.2 incubated at 35 +/- 2 °C for 18 to 24 h. The enrichment was made in Tetrationate (TT) broth and Rappaport - Vassiliadis Modified (RV) broth incubated at 35 °C and 42 °C / 24 h, respectively. For isolation of *Salmonella* were used the selective media of Hektoen Enteric agar (HE), Xilose Lysine Deoxycholate agar (XLD) incubated at 35 °C / 24 h and Bismuth Sulfite

agar (BS) at 35 °C/ 48 h. Colonies with typical characteristics of *Salmonella* were transferred to Brain Heart Infusion Agar (BHI) 35 °C/ 24h and then were preserved in tubes with 20 % glycerol.

2.3 Molecular identification

For the confirmation of typical colonies of *Salmonella* it was made the PCR specific group method in the Microbial Resources Division at CPQBA - UNICAMP. The isolates were reactivated in BHI broth and incubated at 35 °C/ 24 h and transferred to McConkey agar to verify the purity. Then, one colony was transferred to nutrient agar at 35 °C/ 24h. Boiling technique was used for DNA extraction, suspending five colonies in 50 µl of sterile water miliQ. The tube was incubated in thermocycler (Eppendorf, Mastercycler) at 95 °C for 3 min for cell lysis.

For identification of the conserved region *inv*A were used the primers Salm3 (5'-GCTGCGCGCGAACGGCGAAG-3') and Salm4 (5'-TCCCGCCAGAGTTCCCATT-3'). The reaction mixture containing 1 x PCR buffer (Invitrogen), 2.5 mM magnesium chloride, 200 µM of each dNTP, 20 pmol of each primer, 1 U Taq DNA polymerase (Invitrogen). Amplification conditions were: denaturation 95 °C for 5 min, 35 cycles of 95 °C for 1 min, 65 °C for 1 min and 72 °C for 1.5 min and final extension of 72 °C for 10 min [14]. It was visualized in agarose gel at 1.2 % TBE 1X electrophoresed for 30 min under conditions of 5V for cm.

The isolates that showing band with specific group primers were sequenced amplifying the homologous regions of rDNA 16S of bacteria[15], using primers 10f (5' CCGCTAATTTCAAAAACTAAG 3') and 1100R (5'AGGGTTGGGGTGGTTG 3'). The PCR products were purified using mini-columns (GFX PCR DNA and Gel Band purification kit, GE Health Care) and reactions of sequencing were performed by kit BigDye® Terminator v3.1 Cycle Sequencing Kit (Life Technologies) and subjected to the sequencing using the automatic sequencer ABI3500XL Series (Applied Biosystems). For the contig assembly was used the program Bioedit version 7.2.5 and compared with the sequences of type organisms in the databases of Genbank (http://www.ncbi.nlm.nih.gov/) and RDP Release 10 (http: / /rdp.cme.msu.edu/).

3. Results and discussion

The Salm3 and Salm4 primers amplified a DNA fragment of approximately 368 bp for the samples corresponding to *Salmonella* spp used as positive control and two samples from non-irradiated samples. However, they were not isolated or identified other *Salmonella* spp. isolates. With the same primers as other bacteria and *Enterobacter hormaechei* and *Klebsiela pneumoniae* were amplified positioned with one to two bands between 400 and 600 bp, respectively. These microorganisms were recovered in the control without irradiation and the 2kGy irradiation (Figure 1).

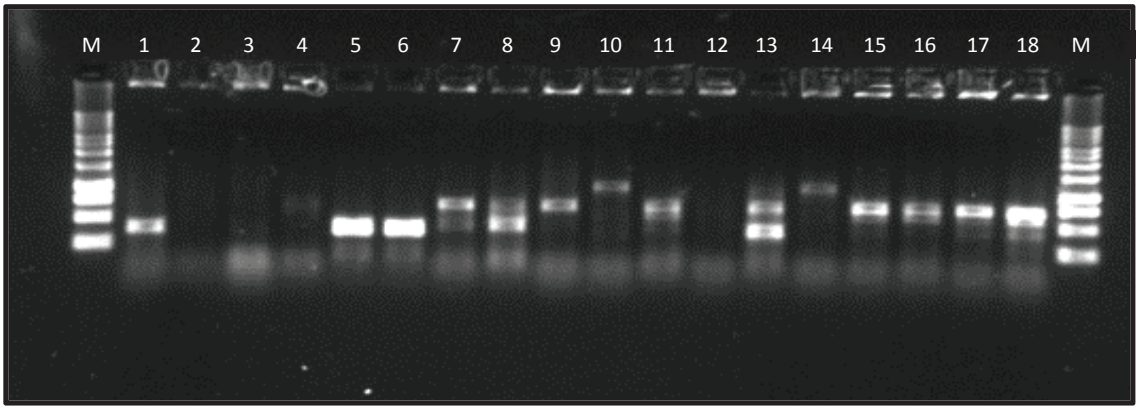

Fig 1: Agarose gel electrophorese of the PCR products DNA from isolated *Salmonella* (invA) colonies. Lane M: Molecular weigth (1Kb plus DNA ladder); Lane 1, 2, 3: *Salmonella* ATCC 13076 positive control; *Pseudomonas* ATCC 13388; *E. coli* ATCC 11775, respectively; Lane 5-6: **Amplification 368 pb *Salmonella* enterica ATCC 1331;** Lane 7, 9, 10, 13, 14, 15 Amplification *Enterobacter hormaechei* CIP 103441; Lane 11, 16, 17, 18 Amplification *Klebsiella pneumoniae* DSM 30104; Lane 4 and 12 not amplification.

Gamma radiation is a technique used as a method for sterilizing surfaces and food. However, it has been documented that in some cases has not been obtained *Salmonella* control at different levels of irradiation [12]. In other studies, it was observed that irradiation of cocoa products (cocoa husks) at 5 kGy dose microbiano[16] controlled growth.

4. Conclusion

It showed that gamma irradiation dosage at 3 and 5 kGy were effective to control the growth of enterobacteria and *Salmonella* cocoa samples. Minimum dose of 2 kGy is not sufficient to prevent possible proliferation of enterobacteria.

Acknowledgements

National Council for Scientific and Technological Development (CNPq), the financier of studies and projects.

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Presented at ICPMF9 (Set 8-12, 2015 – Rio de Janeiro, Brazil) as paper PMOD2015_029